Bull. Environ. Contam. Toxicol. (2000) 65:1-7

DOI: 10.1007/s001280000086

Bulletin of Environmental Contamination and Toxicology

Improved Rapid Analytical Method for the Urinary Determination of 3,5,6 Trichloro-2-Pyridinol, a Metabolite of Chlorpyrifos

B. A. MacKenzie,[1] C. A. F. Striley,[1] R. E. Biagini,[1] L. E. Stettler,[1] C. J. Hines[2]

[1] Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Division of Applied Research and Technology, 4676 Columbia Parkway, Cincinnati, OH 45226, USA
[2] Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Division of Surveillance Hazards Evaluation and Field Studies, 4676 Columbia Parkway, Cincinnati, OH 45226, USA

Received: 15 February 2000/Accepted: 11 May 2000

Urinary immunoassays have been used as screens for occupational exposure to a variety of compounds, including pesticides (Feng et al. 1994; Biagini et al. 1995; Mastin et al. 1998). Immunoassays are generally quite sensitive, specific and accurate. However, biotransformation of xenobiotics may lead to urinary excretion of metabolites, which, in some cases, may lead to modulation of immunoreactivity yielding an over/under estimation of apparent body burdens, presumably from enhanced/inhibited competition by metabolic conjugates (Biagini et al. 1995). Hence, immunoassays for biological monitoring must be carefully evaluated to identify potential interferences from conjugates and other metabolites.

Our laboratory is currently investigating immunochemical biological monitoring methods for various organophosphate pesticides. Of particular interest is a method for chlorpyrifos (CP - Figure 1 [A]), which is used to control a broad-spectrum of insects in the home and in agriculture. CP is one of the most widely used insecticides in the United States, with approximately 9-13 million pounds applied for crop protection and 2-4 million pounds applied for nonagricultural uses in 1995 (U.S. EPA 1997).

Studies in channel catfish (Barron et al. 1991) and rats (Nolan et al. 1987) have shown the major urinary metabolite of CP to be the glucuronide conjugate (Figure 1 [B]) of 3,5,6-trichloro-2-pyridinol (TCP- Figure 1[C]). Urinary TCP has been used as the marker of exposure in studies investigating occupational and non-occupational exposures to CP (Fenske and Elkner 1990; Chang et al. 1996; Nolan et al. 1984). Acid hydrolysis has been used to liberate TCP from urine conjugates for subsequent urinary determinations by gas chromatography (Fenske and Elkner 1990; Nolan et al. 1984), gas chromatography-negative ion chemical ionization mass spectrometry (Ormand et al. 1999), high performance liquid chromatography (Chang et al. 1996) and immunoassay (Shackelford et al. 1999). Recovery of TCP has been shown to be increased in urine samples incubated with glucuronidase, suggesting that humans, like rats, excrete TCP as a glucuronide (Nolan et al. 1984). Enzymatic hydrolysis with β−glucuronidase was used by Hill et al. (1995) in conjunction with GC/MS/MS to measure 12 pesticide residues, including TCP, in urine. While a 17 hour β−glucuronidase incubation at 37 °C was used by Hill et al. (1995) a study by Simonsson, et al. (1995) indicated that a much shorter incubation time (at least 30 min) at room temperature was successful in increasing the

Correspondence to: B. A. MacKenzie

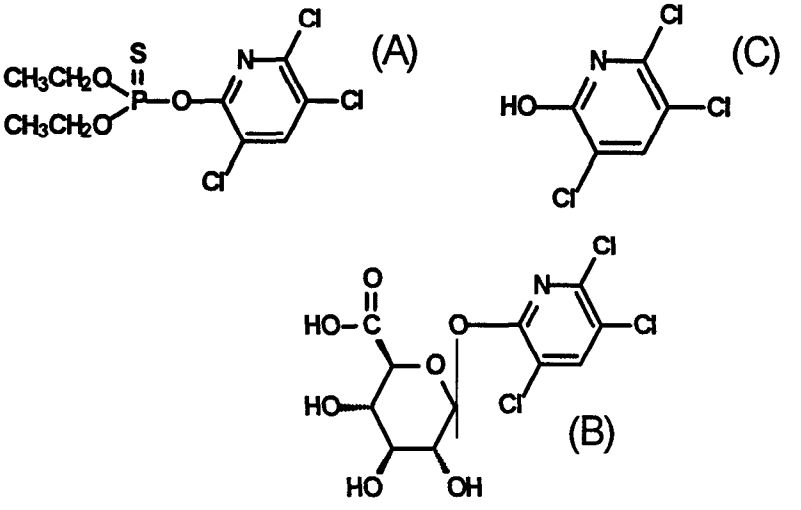

Figure 1. Structure of chlorpvrifos and some of its metabolites.

sensitivity of an immunoassay for urinary benzodiazepine without reducing the specificity of the method. In the present investigation, we describe a method using β-glucuronidase treatment of urine from individuals occupationally exposed to CP and a simple particle-based immunoassay. This method yields results correlating extremely well with a more elaborate, multi-step conventional method, acid hydrolysis followed by analysis for TCP by gas chromatography with mass selective detection (GC-MSD).

MATERIALS AND METHODS

The present investigation was part of a field study of workers who commercially apply CP-containing termiticides. A total of 285 urine samples were submitted from the field, including 28 quality control (QC) samples submitted blindly. These QC samples, prepared using pooled urine from volunteers, were fortified with TCP (ChemService, Inc., West Chester, PA) at concentration levels of 0, 19.7, 222, and 2464 ng/mL. Also included as samples submitted from the field were blind splits of 33 individual worker urine samples. All samples were analyzed for TCP by a commercial laboratory (Pacific Toxicology Laboratories, Woodland Hills, CA) using a proprietary unpublished method. A randomly selected subset of the field samples, 94 worker urines, 16 blind splits, and 14 QC urine samples (124 total) were analyzed at the NIOSH laboratory, using a commercial TCP immunoassay after β-glucuronidase treatment.

For immunoassays, acommercially available enzyme linked immunosorbent assay (ELISA) kit (Trichloropyridinol RaPID® Assay kit, Strategic Diagnostics Inc., Newark, DE), primarily designed for the analysis of TCP in water, was modified for urinary TCP determinations. Shackelford et al. (1999) used this same kit for urinary TCP analysis after acid hydrolysis and an extensive sample clean up procedure. Our primary modifications to the kit procedure were the preparation of standard curves in urine diluted with phosphate-buffered saline (PBS - 0.02M phosphate buffer, pH 7.4, containing 0.9% NaCl), the addition of β−glucuronidase (β−D-glucuroniside glucuronosohydrolase, E.C. 3.2.1.31, from

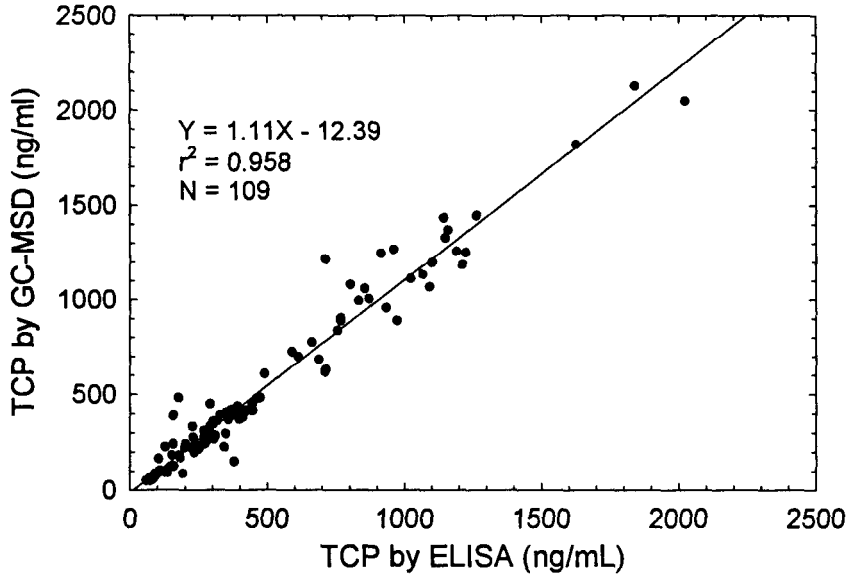

Figure 2. Comparison of urinary TCP (GC-MSD vs. ELISA).

Escherica coli K-12 [RNAse negative], Boehringer Mannheim, Indianapolis, IN), and an additional incubation period. In experiments using urine from workers in this study, the incubation times and amounts of β−glucuronidase were varied systematically, and increases in TCP (compared to non-β-glucuronidase-treated urines) reached a plateau with an addition of 20 µL β−glucuronidase and a 30 min incubation time (data not shown).

Stored, frozen urine samples were thawed, vortexed, and an aliquot at the target dilution prepared. β-glucuronidase (20 µL) was pipetted into the bottom of the tube provided with the kit, and 250 µL of diluted sample or standard was added. The mixture was vortexed and incubated at room temperature for 30 min. The samples and standards were then analyzed as per kit instructions.

The RaPID® kit is a competitive ELISA method where enzyme (horseradish peroxidase) labeled-TCP and TCP from a sample or standard are allowed to compete for TCP-specific antibodies attached to paramagnetic particles. After incubation, a magnetic field is applied to the particles (Magnetic Particle Separation Rack, Strategic Diagnostics), which are subsequently washed to remove unbound reactants. The amount of bound conjugate is revealed by the addition of a color reagent consisting of substrate (hydrogen peroxide) and a chromogen (3,3',5,5' tetramethylbenzidine). After incubation, the reaction is stopped and the optical density (OD) at 450 nm measured in a photometer (RPA-1 RaPID® Analyzer, Strategic Diagnostics).

A stock solution of TCP, at a concentration of 10,000 ng/mL in PBS, was used to prepare standards in PBS-diluted pooled urine. PBS-diluted urine TCP standards of 0.5, 1.0, and 5.0 ng/mL were used as calibrators for standard curves. Levels of TCP were estimated by interpolation from the linear regression equation of the standard curve of log,,-logit

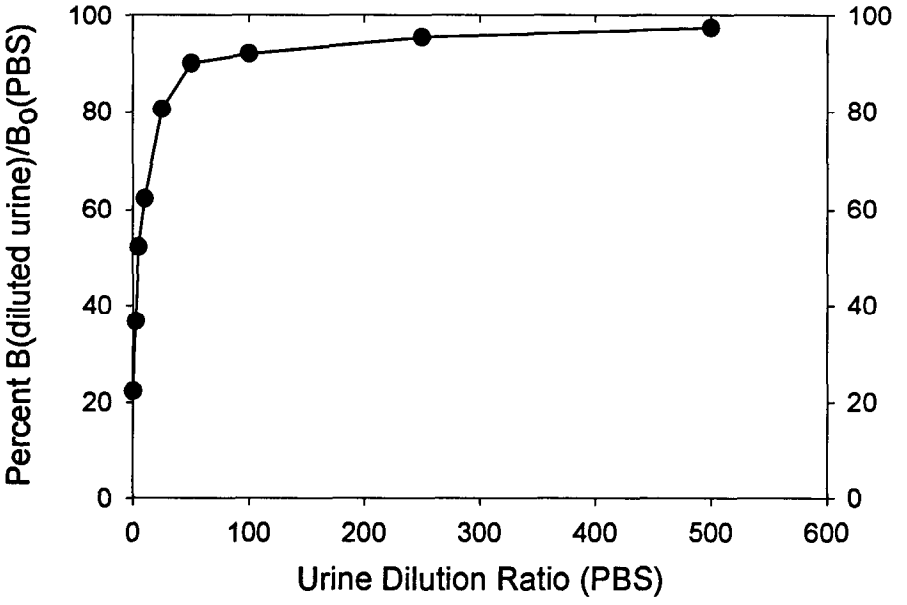

Figure 3. Matrix effect of urine on TCP ELISA compared to PBS.

transformed B/B_0 data, where B = the mean OD for each TCP standard and B_0 = the mean optical density measured for the diluted urine blank. Preliminary analyses had shown urine from the CP-exposed workers yielded results 50-1000 times higher than the range of the standard curve. Consequently, worker urines were initially run at a 1: 100 dilution in PBS. Samples with results ≥5.0 ng/mL were reanalyzed at a dilution of 1: 1000 in PBS to bring their results into the range of the standard curve. For initial results ≤ 0.5 ng/mL, the samples were analyzed at a 1:50 dilution. Note: All standards used to prepare calibration curves were prepared at the same dilution levels as the worker urine samples; i.e., 1:50, 1:100, or 1:1000 in PBS. All samples in the same batch were run in duplicate. A PBS-diluted urine TCP standard at 2.0 ng/mL was added as a sample to each run to serve as an inter-batch control.

For the GC-MSD analyses, the stored, frozen urine samples were brought to 35°C in a water bath to dissolve any solids. After thorough mixing by shaking, a 1-mL aliquot of urine from each sample was fortified with 10 µL of 20 µg/L $^{13}C_2{}^{15}N$-labeled-3,5,6-TCP (in acetone) as an internal standard and 100 µL of concentrated (12*N)* hydrochloric acid. The sample vials were sealed with Teflon™-lined caps and vortexed for 5-10 sec. Samples were then hydrolyzed in an 80°C water bath for 60 min. After hydrolysis, samples were brought to room temperature and 1 mL each of 20% aqueous sodium chloride and 1-chlorobutane was added to each sample. Sample vials were then capped and vortexed for 10 minutes. After vortexing, the samples were centrifuged for 5 minutes at 2700 rpm. The top 1-chlorobutane layer was transferred to a 2-mL autosampler vial and 100 µL of the derivatizing agent N-methyl-N-(tert-butyldimethylsilyl)-trifluoro-acetamide (MTBSTFA)

was added to each autosampler vial. The samples were vortexed for 5-10 sec and placed in an oven at 60°C for 60 min, and analyzed for TCP by gas chromatography with mass selective detection as described below.

AHewlett-Packard 6890 gas chromatograph equipped with aHewlett-Packard 5973 mass selective detector (Wilmington, DE) and a Durabond-17, 30-m x 0.18-mm i.d., 0.3 film thickness fused silica capillary column (J&W Scientific, Folsom, CA) was used for determination ofTBDMS derivative of TCP. The injection and interface temperatures were 280°C. An initial oven temperature of 80°C was held for 1 min, then ramped at 10°C/min to 180°C, followed by a second ramp at 20°C/min to 280°C, where the temperature was held for 2 min. The carrier gas was helium. The mass selective detector used electron impact ionization.' Ions at m/z 254 (quantitation) and m/z 256 (confirmation) were monitored for the *tert*-butyldimethylsilyl (TBDMS) derivative of TCP, and an ion at m/z 261 for the derivatized internal standard (TBDMS derivative of $^{13}C_2{}^{15}N$-labeled TCP). The GC-MSD method limit of detection (LOD) was 2.0 ng/mL.

Statistical tests were performed with SPSS for Windows, version 9.01 (SPSS, Inc, Chicago, IL). A type 1 error level of $P<0.05$ was considered statistically significant. Correlation between GC-MSD and ELISA results were performed using simple linear regression techniques.

RESULTS AND DISCUSSION

Neat urine interferes with the ELISA to seriously hinder its sensitivity and dynamic range. This urine matrix effect was evaluated by determining the $\%B/B_0$ of varying PBS dilutions of blank urine compared to PBS. As can be seen in Figure 2, there is a steep increase in % B/B, up to a urine dilution of 1:50 (urine:PBS), where a plateau was reached upn further dilution. Results of simple linear regression analyses of ELISA TCP standard curves prepared in water or PBS-diluted urine are shown in Table 1.

Table 1. Results of simple linear regression analyses of ELISA TCP standard curves in water and in PBS-diluted urine matrices.

Matrix	Slope	Y-Intercept	r^2
1:50 Diluted Urine	-2.24	0.84	0.994[A]
1:100 Diluted Urine	-2.30	0.96	0.996[A]
1:1000 Diluted Urine	-2.25	0.99	0.999[A]
Water	-2.44	1.18	0.998[A]

[A] $P<0.001$

The least detectable dose (LDD) of the modified ELISA assay, defined as 90% B/B_0, was 0.25 ng/mL, with a limit of quantitation [LOQ (3 x LDD)] of 0.75 ng/mL. Because of the urine matrix effect, a dilution of at least 50:1 was necessary for analysis of the field samples. Hence, the practical LDD for the field urine samples was 12.5 ng/mL (50 times the modified ELISA assay LDD), with an LOQ of 37.5 ng/mL. The mean coefficient of variation (CV) for all duplicate analyses within a batch was 1.88% ± 0.15% (SEM). The mean recovery for the 2 ng/mL PBS-diluted urine TCP inter-batch quality control

standards was 2.16 ± 0.05 ng/mL (N=6, range: 1.96-2.32 ng/mL, CV = 5.80%). TCP was found in the urine of 82% of 993 U.S. adults sampled from the general population (Hill et al. 1995a) with a mean concentration of 4.5 ng/mL (median =3.0 ng/mL). TCP also was

Table 2. Summary of quality control (QC) recoveries by ELISA and GC-MSD.

QC Sample Concentration (ng/mL)	ELISA Percent Recovery ± Standard Deviation (N= No. of Samples)	GC-MSD Percent Recovery ± Standard Deviation (N= No. of Samples)
26.2[A]	-------[B] (N=4)	95.7 ± 6.1 (N=7)
228[A]	103.8 ± 7.3 (N=3)	98.8 ± 6.7 (N=7)
2470[A]	84.8 ± 16.6 (N=3)	90.9 ± 4.8 (N=7)

[A]QC concentration adjusted by adding the average of GC-MSD results for seven QC samples having no added TCP (6.5 ng/mL).
[B] Concentration below the practical urine LOQ.

present in our QC samples containing no added TCP [mean concentration of 6.5 ± 1.5 (standard deviation) ng/rnL TCP as determined by the GC-MSD analysis]. Consequently, this mean "background level" was used to adjust the concentrations of QC samples to reflect the added and pre-existing concentrations of TCP. The recoveries for the QC samples are shown in Table 2. For the split samples, the mean CV was 10.3% (range = 0 to 46.4%; N=16) by ELISA and 3.1% (range = 0.1 to 16.3%; N=33) by GC-MSD.

When correlation between the two methods for all samples having TCP concentrations above the LOQ for both analytical methods (n=109) was investigated using simple linear regression, a significant ($P<0.0001$; $r2= 0.958$) association was observed. The relationship between the two methods was: GC-MSD results = 1.11 x ELISA results - 12.39 ng/mL TCP (Figure 3). These results strongly suggest both methods were measuring the same analyte (TCP).

The ELISA kit used for urinary TCP analysis was designed for use in a water matrix, and the urine matrix clearly affects its utility. While sample clean-up procedures might eliminate or reduce the matrix effects, the time required for these steps would negate the advantage in analysis time and cost for the ELISA compared to the instrumental method. Consequently, we chose to use dilution with PBS to minimize matrix effects. The net effect of dilution was to increase the *practical* LOQ of the ELISA procedure to 37.5 ng/mL for undiluted urine, compared to an LOQ of 6.0 ng/mL for the GC-MSD method. Hence, our procedure was not suitable for measuring urinary levels of TCP <37.5 ng/mL. Despite this apparent shortcoming, the ELISA LOQ for diluted urine was well below the TCP levels found in all but one of the samples from the occupationally-exposed individuals in the present work. The GC-MSD method used in the present work requires a l-hr acid hydrolysis step, extractions, and derivatization (1 -hr) followed by analysis with expensive, sophisticated instrumentation. The ELISA procedure requires no sample clean-up and the enzymatic hydrolysis step takes only 30 minutes. The ELISA assay is run directly on the hydrolysate and takes less than 1 hour. The high correlation between methods and the slope of the correlation curve for the ELISA vs. GC-MSD approaching unity, indicates the

β-glucuronidase-hydrolyzed ELISA procedure is a viable adjunct to classical instrumental methods for urinary TCP, particularly in screening for occupational exposure to CP.

Acknowledgments. We thank Mrs. Shirley Robertson for her excellent technical assistance and also express our appreciation to the workers and companies involved for their willingness to participate in this study.

REFERENCES

Barron MG, Plakas SM, Wilga PC (1991) Chlorpyrifos pharmacokinetics and metabolism following intravascular and dietary administration in channel catfish. Toxicol Appl Pharmacol 108:474-82

Biagini RE, Henningsen GM MacKenzie BA, Sanderson WT (1995) Urinary bio-monitoring for alachlor exposure in commercial pesticide applicators by immunoassay. Bull Env Contam Toxicol 54:245-250

Chang MJW, Lin CY, Lo LW, Lin RS (1996) Biological monitoring of exposure to chlorpyrifos by high performance liquid chromatography. Bull Env Contam Toxicol 56:367-374.

Feng PC, Wratten SJ, Horton, SR Sharp, CR, Logusch, EW (1990) Development of an enzyme-linked immunosorbent assay for alachlor and its application to the analysis of environmental water samples. J Agric Food Chem 38: 159-163

Hill RH, Shealy DB, Head SL, Williams CC, Bailey SL, Gregg M, Baker SE, Needham LL (1995) Determination of pesticide metabolites in human urine using an isotope dilution technique and tandem mass spectrometry. J Anal Toxicol 19:323-329

Hill RH, Head SL, Baker S, Gregg M, Shealy DB, Bailey SL, Williams CC, Sampson EJ, Needham LL (1995a) Pesticide residues in urine of adults living in the United States: reference range concentrations. Environ Res 71:99-108

Mastin JP, Striley CAF, Biagini RE, Hines CJ, Hull RD, MacKenzie BA, Robertson SK (1998) Use of immunoassays for biomonitoring of herbicide metabolites in urine. Analytica Chimica Acta 376:119-124

Nolan RJ, Rick DL, Freshour N L, Saunders JH (1984) Chlorpyrifos: pharmacokinetics in human volunteers. Toxicol Appl Pharmacol 73:8-15

Nolan RJ, Drygza MD, Kastl PE (1987) Chlorpyrifos: distribution and metabolism in the Fischer 344 rat. Toxicologist 7:45

Ormand JR, McNett DA,Bartels MJ(1999) Semiautomated preparation of3,5,6-trichloro-2-pyridinol in human urine using a Zymate XP laboratory robot with quantitative determination by gas chromatography-negative-ion chemical ionization mass spectrometry. J Anal Toxicol 23 :35-40

Shackelford DD, Young DL, Mihaliak CA, Shurdut BA, Itak JA (1999) Practical immunochemical method for determination of 3,5,6-trichloro-2-pyridinol in human urine: applications and considerations for exposure assessment. J Agric Food Chem 47:177-182

Simonsson P, Lidén A, Lindberg S (1995) Effect of β−glucuronidase on urinary benzodiazepine concentrations determined by fluorescence polarization immunoassay. Clin Chem 41:920-923

U.S. EPA (1997) Pesticide industry sales and usage: 1994 and 1995 Market Estimates. Office of Prevention, Pesticides and Toxic Substances: Washington, DC, pp. 19-20